Binding of Antioxidants by Milk Proteins

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Abstract

The binding of esters of gallic acid to milk proteins increases with increasing alkyl chain length. Chain branching decreases binding but changes in temperature, salt concentration, and heat treatment of the proteins have little effect. Butylated hydroxy anisole binds to the same extent as propyl gallate. Calcium-free milk proteins bound less than natural milk proteins. Binding is reversible and at low concentrations the amount of antioxidant bound is a linear function of the concentration of free antioxidant. The proteins of milk would thus compete with the fat phase for antioxidant.

Introduction

Stabilization of the initial flavor of whole milk powders produced by various spray (2) and vacuum (5) drying techniques is one of the objectives of research in the dairy products laboratory. Although stability against flavor change by oxidation can be enhanced by packaging in inert gas or heating milk before drying, certain objections to these procedures arise. Packaging in inert gas is expensive and a strongly cooked flavor characterizes powders from milk heated above pasteurization requirements. Attempts have been made to avoid these problems with antioxidants but with limited success (6).

Many antioxidants work well when added to single component foods such as cooking oils but perform poorly in heterogeneous systems such as whole milk powder. The antioxidant should presumably reside either in the fat or at the fat globule surface for adequate protection of these powders. The distribution of the various antioxidants in such a system will probably depend on their interactions with the milk constitutents, the method of incorporation, and the method of powder manufacture.

The actual distribution of added antioxidants in fluid and dried milk is not known. Work has been performed in the authors' laboratory on the partitioning of the esters of gallic acid, butylated hydroxyanisole (BHA) and nordihydroguaiaretic acid (NDGA) in butteroil/water model systems (1). Results indicated that each antioxidant was characteristically distributed between the oil and water phases with BHA showing the highest preference for the oil phase. Increasing the alkyl chain length of the gallates caused increased preference for the oil phase. The interaction of these antioxidants with the proteins of milk would influence their distribution. The binding of ions and small molecules by proteins is well documented (7). The interaction of long chain detergents such as sodium lauryl sulfate with proteins has also been studied (4).

All of the antioxidants we have studied are molecules containing both polar and nonpolar groups hence either polar, nonpolar, or both types of interactions with milk proteins might be expected. The main objective of our work was to determine, quantitatively, the interactions of antioxidants with milk proteins. Such data in combination with the measured partition coefficients of antioxidants (1) would be a basis for predicting their distributions in milk products.

Materials and Methods

The antioxidants used in this work have been described (1). The synthesis of labeled propyl gallate has been reported (1). Synthesis of the C-14 labeled hexyl gallate was performed similarly with labeled hexanol from the Mallinckrodt Chemical Company.[1] After multiple recrystallizations from distilled water, the labeled hexyl gallate melted at 92 C, agreeing with the literature. The dialysis tubing was from Arthur H. Thomas Company.[1] The skimmilk was from a herd of Holstein cows maintained by the Department of Agriculture at the Beltsville, Maryland Experimental Station. The salt solution approximating the ionic composition of milk has been described by Jenness and Koops (3). The milk proteins were prepared by dialysis of about one liter of skimmilk against two 10 to 15 liter batches of the salt solution for two days. In some cases raw skimmilk was used, however, most work was

[1] Mention of brand or firm names does not constitute an endorsement by the Department of Agriculture over others of a similar nature not mentioned.

performed with skimmilk pasteurized at 63 C for 30 min. Binding studies were also performed on whey proteins and calcium-free skimmilk. Acid or sweet whey was treated like skimmilk to obtain solutions of whey proteins. Removal of calcium from skimmilk was with sufficient sodium oxalate to completely precipitate calcium oxalate (about 50 mmoles of oxalate per liter of skimmilk). Oxalated milk was then spun in a centrifuge at 4,000 rpm for 15 min after which the supernatant was decanted from the calcium oxalate residue. Excess oxalate was then removed from the supernatant by dialysis against two changes of the Jenness and Koops salt in which the calcium ion was replaced with a molar equivalent of magnesium from MgO.

The binding studies were performed using standard equilibrium dialysis. Sacs of dialyzed skimmilk were placed in antioxidant (exterior phase) solutions of known volume and concentration and rotated gently on a platform until they were equilibrated. Equilibration for 24 hr was sufficient. The antioxidant concentration in the exterior phase was then determined and compared to expected results from volumetric dilution alone. A lower antioxidant concentration indicated that binding of the antioxidant to the protein was occurring. Protein in the interior phase was determined by the micro-Kjeldahl method using factor 6.38 to convert nitrogen to protein. The antioxidant solutions were analyzed either by photometric analysis or by liquid scintillation counting of C-14 labeled propyl and hexyl gallate. For photometric analysis solution absorbances were determined at 286 mμ for BHA and 270 mμ for the gallates.

When volumes and concentrations of various phases are known, bound antioxidant can be determined from the relationship,

$$m = C_s V_e - C_f(V_e + V_i) \qquad [1]$$

where m = the number of moles of antioxidant bound to protein.

C_s and C_f = the antioxidant concentrations (moles/liter) of the starting and final (exterior) solutions.

$V_e + V_i$ = volumes in liters of the exterior (protein free) and interior (protein) solutions.

Initially, all the antioxidant is in the external phase, hence $C_s V_e$ represents the total amount of antioxidant added to the system. After equilibration, the total amount free in the aqueous phase is $C_f(V_e + V_i)$. The difference in these two terms was the amount bound by milk proteins. V_i was taken to be equal to the interior water without correcting for the partial specific volume of the protein. Since dilute solutions (2 to 3% protein) were used, no serious error was introduced.

In the aforementioned formula where the final concentration is the expected value by volumetric dilution alone, i.e., $C_f = C_s\ V_e/(V_e - V_i)$ the value of m is zero indicating no binding.

By rearranging Expression 1 and adding a term for the weight of the protein, we arrive at the expression to calculate our binding data.

$$M = m/w = C_s[V_e - (C_f/C_s)(V_e + V_i)]/W \qquad [2]$$

where M = moles of antioxidant bound per gram of protein.

W = weight of protein in grams.

The other terms are defined in Equation 1. For calculation, the ratio C_f/C_s in Equation 2 was replaced by the appropriate analytical response, either absorbance ratios from the photometric analyses or counts per minute ratios from the analyses of labeled molecules.

For the study of binding reversibility with propyl gallate, first the usual equilibrium dialysis was performed. Then the sacs containing protein were removed from the exterior solution, washed lightly in distilled water, dried with a paper towel and immersed in known small volumes of distilled water in test tubes and rotated gently on a dialysis platform overnight. The exterior phase was then analyzed for propyl gallate, which became the new "free" concentration. From these values and from volumes of the various phases and amounts of antioxidant taken up in the original binding, the bound antioxidants were determined.

Results

The binding of propyl gallate to proteins in normal and calcium-free milk is illustrated in Figure 1 where at low concentrations the curves can be approximated by straight lines. The effect of chain length of alkyl gallates on the binding at low concentrations is given in Figure 2. Whey proteins (not shown) bound propyl and hexyl gallate to the same extent (per gram of protein) as did the complete mixture of milk proteins. Figure 3 shows reversibility of the binding process. The filled circles represent the results of the first uptake of antioxidants by milk proteins. It is these points through which the solid line is drawn. The protein solutions containing the bound antioxidant are then immersed in measured volumes of distilled water. Some antioxidant is removed and a new equilibrium is established (open circles). Desorption occurs along the original

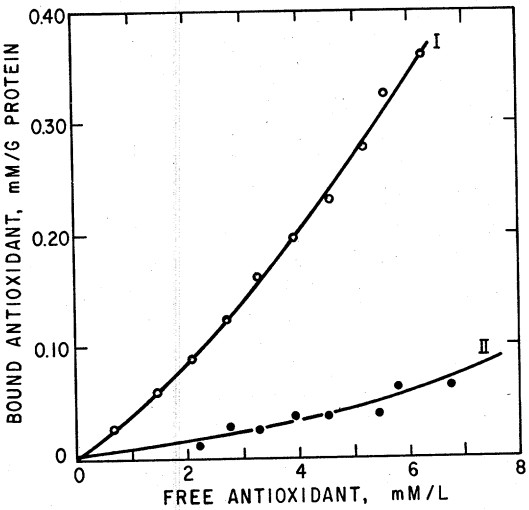

FIG. 1. Binding of propyl gallate by normal (Curve 1) and calcium free (Curve 2) milk proteins.

curve showing that binding is reversible. Any irreversible binding would have resulted in the new points (open circles) lying above the solid line. Table 1 gives the slopes of the curves for butylated hydroxyanisole (BHA) as well as for the alkyl gallates.

Both propyl and hexyl gallate passed freely through the dialysis membrane and were distributed evenly throughout a protein-free system. There was no evidence for binding to the membrane.

Discussion

At low concentrations the curves of Figure 1 closely approach straight lines passing through the origin. These limiting slopes are defined by the equation,

$$M = b\,C_f \qquad [3]$$

TABLE 1. Binding of antioxidants to milk proteins.

Antioxidant	Binding coefficient b
Ethyl gallate	0.023
Propyl gallate	0.040 (0.003)[a]
Butyl gallate	0.084
Amyl gallate	0.17
Hexyl gallate	0.36
Isopropyl gallate	0.034
Butylated hydroxyanisole (BHA)	0.04

[a] Mean deviation of 5 determinations; the other values are slopes of the binding curves of Figure 2.

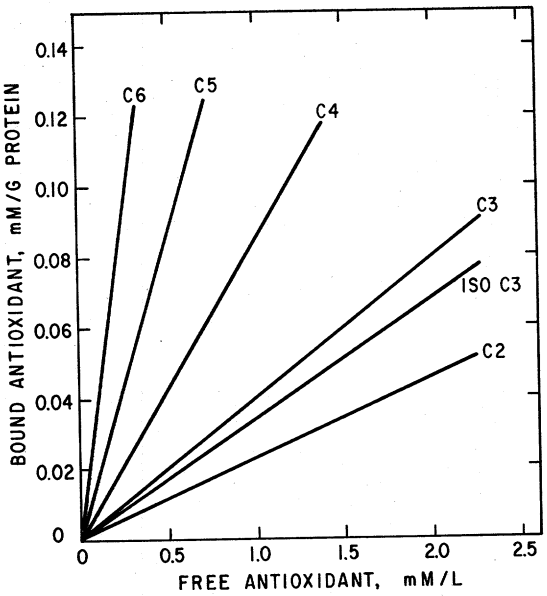

FIG. 2. Binding of alkyl gallates by milk proteins. C2 ethyl gallate through C6 hexyl gallate and iso C3 isopropyl gallate.

where b is the slope and M and C_f have the same meaning as in Equation 2. The data points have been omitted from Figure 2 for clarity, however, they fit the limiting slopes closely. Establishing values of b thus uniquely defines the quantitative relations between the free and bound antioxidants over the concentration range.

The binding coefficients of the alkyl gallates exhibited a twofold increase for each addition of a methylene group. Chain branching ap-

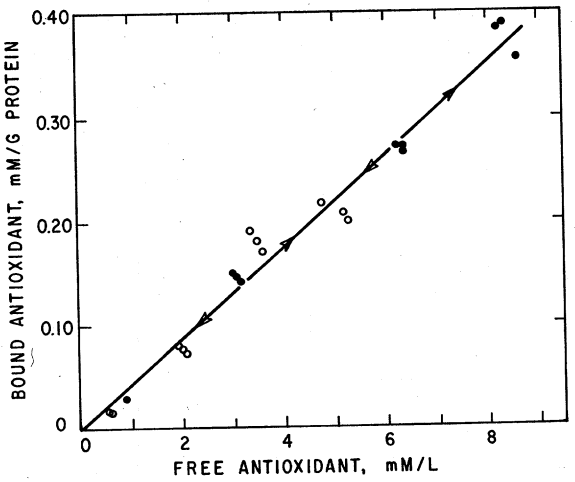

FIG. 3. Binding reversibility of propyl gallate to milk proteins. Filled circles binding (sorption) step, open circles desorption step.

parently inhibits binding to some degree as shown by comparing isopropyl and propyl gallate. These observations indicate nonpolar rather than polar protein-antioxidant interactions. Removing calcium from the milk proteins gave the expected decreased turbidity due to breakup of casein micelles. The resulting mixture exhibited markedly reduced binding of propyl gallate, possibly due to a reduction in the nonpolar environment available for interaction.

No effect of temperature between 4 and 37 C and milk salt concentration varying from the single strength of normal milk to about a tenth that value was noted on the binding of propyl gallate to milk proteins. Both raw and pasteurized milk bound propyl gallate equally.

The milks continued to bleed a small amount of material through the dialysis membranes which absorbed at wavelengths used for optical analysis. For the gallates this absorbance was rendered negligible by the dilutions necessary for analysis. For BHA the results were somewhat less accurate, yielding only one significant figure for the binding coefficient. No reproducible values for the binding of NDGA could be obtained due to its limited solubility. Attempts to account for the bleeding of the proteins by a background correction were unsuccessful.

Antioxidant concentrations generally used in foods are much lower than the upper values in Figure 1. For example, whole milk powder containing .01 g propyl gallate per 100 g powder, when reconstituted to single strength milk, will contain about 60 μmoles gallate per liter. In this concentration range and considerably above, a linear relationship between the amount bound and the concentration of the unbound (free) species existed.

Significant implications concerning the distribution of added antioxidants in milk can be drawn from our results. Previous work has shown that each antioxidant establishes a characteristic distribution between the oil and water phases of model systems. In milk, the protein would compete with butteroil for the antioxidant, resulting in less antioxidant in the fat phase of milk than expected from its distribution coefficient in protein-free model systems. The extent of this competition and its effect on the distribution of antioxidants in dairy products is under investigation.

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